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Many of the molecular events that control normal development of the mammary gland are the same events that, when misregulated, result in cancer. Therefore, the understanding of normal developmental processes in the mammary gland is a crucial first step to rational design of therapeutics which target these systems in breast cancer.

The Wnt family of genes, which were first identified for their role in mouse mammary tumorigenesis, initiate a signaling cascade that manifests in the stabilization of β-catenin protein. The proposed experiments are based on the hypothesis that misregulation of this pathway results in an accumulation of stabilized β-catenin, and genes involved in growth, cell death, and cell invasion are upregulated inappropriately, resulting in tumorigenesis.

The proposed experiments use two methods to study β-catenin's direct role in mammary gland development and tumorigenesis. Reconstitution experiments using retrovirally-transduced, stabilized β-catenin and transgenic mice expressing a dominant negative mutant β-catenin (β-cat^{DN}) specifically in the mammary gland provide opposite approaches for study. These two systems will be analyzed for changes in morphology, downstream gene expression, and functional differentiation, comparing gain- and loss-of-function of β-catenin.

Many factors implicated in mammary oncogenesis regulate β-catenin, and β-catenin is clearly involved in tumorigenesis in other organ systems, suggesting a role for β-catenin in mammary oncogenesis. The proposed experiments will characterize β-catenin's direct role in mammary gland development and tumorigenesis.

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Introduction

β-catenin's involvement in Wnt and other signaling pathways and its oncogenic potential in other tissues strongly suggests a role for β-catenin in mammary tumorigenesis. This study proposes to examine the direct role of β-catenin in normal mammary gland development and tumorigenesis through gain- and loss-of-function experiments, using two independent approaches: a mammary gland reconstitution model and a transgenic model.

Key Accomplishments

Task #1:

1. Learned and optimized mammary gland reconstitution method

The mammary gland reconstitution method involves isolating primary epithelial cells from mammary glands dissected from mice, manually and enzymatically digesting the cells into single cell suspension, manipulating the cells in culture, and then injecting the cells back into a recipient animal that has been cleared of endogenous mammary epithelium. I was trained in this method, optimized the specific conditions, and achieved a "take rate" of almost 100% (i.e., 100% of injection sites grow into a reconstituted mammary gland).

2. Cloned stabilized β-catenin and dominant negative β-catenin mutants into a retroviral backbone

A stabilized β-catenin mutant and a β-catenin dominant negative mutant (β-en) were obtained from Pierre McCrea at M.D. Anderson Cancer Center in Houston, TX and were subcloned into a retroviral backbone containing flanking retroviral LTRs. When these constructs are expressed in cells in the presence of retroviral packaging proteins, the β-catenin or β-en protein is packaged into a retroviral particle and secreted from the cell. The virus-containing medium is then collected and used to infect mammary cells.

3. Optimized conditions for retroviral infection of mammary cells

A variety of methods was employed in the attempt to retrovirally infect mammary epithelial cells. Several stable packaging cell lines were tried, including Phoenix, BOSC23, and GPE86 cells, but none of these cell lines produced consistently high retroviral titer. An alternative method was developed, transiently transfecting 293T cells with retroviral packaging genes along with the β-catenin or β-en construct. A variety of packaging proteins was tried as well, including amphitropic and ecotropic envelope proteins. The virus-containing medium was collected from these transfected 293T cells and transferred to cultured mammary cells. A spin infection method was optimized in which the cells are spun (on a plate adapter in a centrifuge) in the presence of the virus-containing medium and polybrene.

The initial characterization of this method was performed on HC11 cells, an established mammary epithelial cell line, and achieved about 80% infection rate. However, the same spin infection method applied to primary mammary epithelial cells yielded a mere 5% infection rate. Literature searches and personal communication with experts in the field produced no solution to this dilemma, as most experiments of this nature are performed on established cell lines. However, established cell lines do

not maintain the ability to reconstitute a normal mammary gland, and therefore infection of primary epithelial cells is required for the proposed experiments.

4. Developed method for enriching primary culture for progenitor cells

The low infection rate of primary epithelial cells poses an obstacle in my planned reconstitution experiments. The mammary gland is clonal in nature, and the literature suggests that an entire cluster of epithelium can originate from a single progenitor cell. If only 5% of the primary cells are infected, the chances are low that a multi-potent progenitor cell will be infected. Therefore, the outgrowth will probably not express the mutant gene.

This dilemma prompted a discussion within our group regarding enriching the primary culture for multi-potent progenitor cells. We have now developed a method for isolating a population of progenitor cells and can apply this method to the retroviral infection experiments. Therefore, we can continue with the proposed reconstitution experiments, infecting a population of progenitor cells with stabilized β-catenin or β-en and characterize their outgrowths.

Task #2:

1. Subcloned WAP-β-en-WAP

The dominant negative β-catenin mutant (β-en) was designed to inhibit the signaling function of β-catenin while not affecting its role in cell-cell adherence. This is significant because all previous analyses of β-catenin function have been based on total gain- or loss-of-function studies and can not distinguish between β-catenin's multiple roles in the cell.

The mutant was constructed by replacing the C-terminal transactivation domain of β-catenin with the repressive domain of *engrailed*. This construct (β-en) was subcloned between a 1 kb fragment of the Whey Acidic Protein (WAP) promoter and an 800 bp fragment of the 3' UTR from the WAP gene. Previous studies in our lab have shown this combination of WAP fragments directs mammary-specific expression of transgenes. This construct was labeled WAP-β-en-WAP (WBW).

2. Generated four transgenic lines

The WBW construct was microinjected into mouse embryos and transferred to a surrogate mother for gestation. After the pups were born, their genomic DNA was screened by PCR, and four pups were found to carry the transgene integrated into their genomes. These four founder animals were bred and their progeny were screened for expression of the transgene.

3. Analyzed expression of WBW transgene

Expression of the transgene was analyzed in lactating glands (predicted time of high transgene expression) by Northern blot, RT-PCR (RNA level) and Western blot (protein level) using antibody to the myc-epitope. However, no expression of the transgene was detected by any method in any of the

four transgenic lines. In addition, whole mount and histological analysis of lactating glands showed no morphological phenotype in the transgenic mice, when compared to non-transgenic littermates.

Discussions of this problem led us to the conclusion that the large β-en cDNA (3.3 kb) was too large to be recognized by the cell as a natural internal exon. Therefore, we could disguise the cDNA as a large terminal exon (found more commonly in nature) by adding an intron upstream of the β-en gene.

4. Subcloned WBKII

The β-en construct was then subcloned into a new transgenic construct containing a 5' intron and a 3' poly-A sequence. This construct (β-en-KCR) was finally subcloned into a vector containing a 1 kb fragment of the WAP promoter. The final construct (WBKII) is composed of the WAP promoter, a 5' intron, β-en, and a poly-A sequence.

5. Generated five transgenic lines

The WBKII construct was microinjected into embryos as described above, and five transgenic lines were identified by PCR screening of genomic DNA. One of the five lines did not transmit the transgene to its progeny, so the remaining four were characterized for expression and phenotype.

6. Whole mount and histological characterization of lactating transgenic lines

Females from each of the four transgenic lines were bred and sacrificed at 1-day-lactation. Their mammary glands were collected, fixed, and characterized either by whole mount analysis or by histology. Both methods of analysis showed significantly decreased lobuloalveolar development in two of the four transgenic lines. This result fit our prediction; since β-catenin signals growth of cells, we expect that the inhibition of β-catenin by β-en would result in decreased growth and development.

Reportable Outcomes

Constructs:	The β-catenin and β-en retroviral constructs will be valuable to our lab and others for future analysis of β-catenin function.
Methods developed:	The retroviral spin infection and reconstitution methods have been optimized and will be valuable to future experiments.
Transgenic mice:	Two WBKII transgenic lines were established which have severe defects in development and lactation.

Conclusions

Though several complications have arisen involving the proposed experiments, we have identified slight modifications in method and technique that are allowing us to circumvent the problems. We are pleased with the results of the transgenic model, and we feel confident that we will complete the remaining tasks on schedule.